Characterization of Three Abnormal Factor IX Variants (Bm Lake Elsinore, Long Beach, and Los Angeles) of Hemophilia-B

Evidence for Defects Affecting the Latent Catalytic Site

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Abstract

Abnormal Factor IX variant proteins were isolated from the plasmas of three unrelated severe hemophilia-B families that had been previously shown to contain functionally impaired molecules immunologically similar to normal Factor IX. The families studied were: (*a*) a patient with markedly prolonged ox brain prothrombin time, designated Factor $IX_{Bm\ Lake\ Elsinore}$ (IX_{BmLE}); (*b*) three patients (brothers) with moderately prolonged ox brain prothrombin time, designated Factor $IX_{Long\ Beach}$ (IX_{LB}); and (*c*) a patient with normal ox brain prothrombin time designated factor $IX_{Los\ Angeles}$ (IX_{LA}). Each variant molecule comigrates with normal Factor IX (IX_N) both in the sodium dodecyl sulfate and in the nondenaturing alkaline gel electrophoresis. All three variant proteins are indistinguishable from IX_N in their amino acid compositions, isoelectric points, carbohydrate distributions and number of γ-carboxyglutamic acid residues. Each variant protein undergoes a similar pattern of cleavage by Factor XIa/Ca^{2+} and by Factor VIIa/Ca^{2+}/tissue factor, and is activated at a rate similar to that observed for IX_N. All of the three variant proteins also react with an anti-IX_N monoclonal antibody that interferes with the binding of activated IX_N(IXa_N) to thrombin-treated Factor VIIIC. However, in contrast to IXa_N, the cleaved IX_{BmLE} has negligible activity (~0.2%), and cleaved forms of IX_{LA} and IX_{LB} have significantly reduced activity (~5–6%) in binding to antithrombin-III/heparin, and in activating Factor VII (plus Ca^{2+} and phospholipid) or Factor X (plus Ca^{2+} and phospholipid)±Factor VIII. These data, taken together, strongly indicate that the defect in these three variant proteins resides near or within the latent catalytic site. This results in virtually a complete loss of catalytic activity of the cleaved IX_{BmLE} molecule and ~95% loss of catalytic activity of the cleaved IX_{LA} and IX_{LB} molecules.

Introduction

Human Factor IX circulates in plasma as a proenzyme of a serine protease, which participates in an early phase of the blood coagulation cascade (1). It is a single chain vitamin K-dependent protein of 416 amino acids (2) and contains 17% carbohydrate by weight (2, 3). The first 12 glutamic acid residues in the amino terminus of the protein are present as γ-carboxyglutamic acid residues (Gla)[1] and are thought to be involved in Ca^{2+} and phospholipid (PL) binding to Factor IX (1, 4, 5). The present evidence suggests that during clotting, Factor IX may be activated by Factor XIa requiring Ca^{2+} (4–6), and by Factor VIIa requiring Ca^{2+} and tissue factor (7). Activation of Factor IX either by Factor XIa or by Factor VIIa involves two steps. In the first step, arginine $(Arg)^{145}$-alanine $(Ala)^{146}$ bond is cleaved, giving rise to a disulfide bond(s) linked two chain intermediate, Factor IX_{α}[2] (2, 6, 8). In the second step, Arg^{180}-valine $(Val)^{181}$ bond is cleaved, giving rise to Factor IXa and release of an activation glycopeptide (2, 6, 8). The primary role of Factor IXa in clotting is to activate Factor X to Factor Xa (1). The physiological activator of this reaction is believed to be a complex of Factor IXa, Ca^{2+}, PL and thrombin activated Factor VIIIC (1). Factor IXa serves as an enzyme for this reaction, and Ca^{2+}, PL, and activated Factor VIIIC serve as cofactors for this reaction (9). Factor IXa also binds to antithrombin-III, and the binding occurs through the active site residue, serine $(Ser)^{366}$, present in the heavy chain of Factor IXa (2, 6). Factor IXa has also been shown to activate Factor VII to Factor VIIa; this reaction requires Ca^{2+} and PL (10).

Factor IX serves an important role in coagulation since deficiency of the coagulant activity of this protein causes a hemorrhagic state commonly known as hemophilia-B (Christmas disease). In most of the patients, the deficiency of the clotting activity is due to the absence of Factor IX molecule; however, in 10–30% of the patients the deficiency of the clotting activity is due to the presence of abnormal variants of Factor IX molecule (11–13). The molecular abnormalities in

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1. *Abbreviations used in this paper:* Gla, γ-carboxyglutamic acid; Factor IX_{BmLE}, $IX_{BmLake\ Elsinore}$; Factor IX_{LB}, Factor $IX_{Long\ Beach}$; Factor IX_{LA}, Factor $IX_{Los\ Angeles}$; Factor IX_N, Factor IX_{Normal}; Im/NaCl/Alb, 0.05 M imidazole-HCl, 0.15 M NaCl, 1 mg/ml bovine serum albumin, pH 7.5; Tris/NaCl/Alb, 0.05 M Tris-HCl, 0.15 M NaCl, 1 mg/ml bovine serum albumin, pH 7.5; PL, phospholipid; $NaDodSO_4$, sodium dodecyl sulfate.

2. The nomenclature used for human Factor IX and its activation products is that of Davie et al. (2, 6). IX, single chain Factor IX (amino acid residues 1 to 416); IX_{α}, two-chain intermediate (IX cleaved at Arg^{145}-Ala^{146}) consisting of a light chain L (amino acid residues 1 to 145 of IX) and a heavy chain H_{α} (amino acid residues 146 to 416); IXa, the two-chain Factor IXa (IX cleaved at Arg^{145}-Ala^{146} and at Arg^{180}-Val^{181}) consisting of a light chain L and a smaller heavy chain H_{β} (amino acid residues 181 to 416). AP represents activation peptide (amino acid residues 146 to 180), which is released upon conversion of IX_{α} to IXa.

these functionally impaired molecules are just beginning to be investigated. Factor $IX_{Zutphen}$ is a genetic variant with an abnormally high molecular weight and appears to have decreased binding for Ca^{2+} (14). Another variant of Factor IX, Factor $IX_{Chapel\ Hill}$, is well characterized. This variant does not undergo normal activation by Factor XIa; during activation only Arg^{180}-Val^{181} bond is cleaved (15, 16). The inability of Factor XIa to cleave Arg^{145}-Ala^{146} bond in Factor $IX_{Chapel\ Hill}$ is due to the substitution of histidine for arginine at position 145 (17).

In this study we isolate and extensively characterize three variant Factor IX molecules. In initial studies (18, 19), these three variant molecules were shown to undergo normal proteolytic cleavages by Factor XIa or by Factor VIIa/tissue factor. The cleaved variant proteins did not activate Factor X, however. The observed lack of activity of the cleaved variant molecules could stem from structural alterations that impaired the function of the active serine site or from structural alterations that impaired the interactions of cofactors namely Ca^{2+}, PL, and activated Factor VIIIC. Comprehensive data presented herein reveal that each of these cleaved variant proteins has a defective active site in the heavy chain of the molecule. All other functional properties of the molecules appear to be normal.

Methods

Patients. All of the patients studied have a serious bleeding problem, normal Factor IX antigen levels (>70%) and negligible Factor IX coagulant activity (≤1%). Three families studied are: (*a*) a patient with markedly prolonged ox brain prothrombin time (~167 s) designated Factor $IX_{Bm\ Lake\ Elsinore}$ (IX_{BmLE}). Because of the prolonged ox brain prothrombin time, this patient in previous studies has been referred to as IX_{Bm} (18) or G.R. (19). Following the recommendation of the International Society of Thrombosis and Haemostasis (July 1981), we have now included the patient's place of birth in correctly naming this variant; (*b*) three patients (brothers) with moderately prolonged ox brain prothrombin time (60 s) designated Factor $IX_{Long\ Beach}$ (IX_{LB}). This variant has been previously referred to as R.S. (19); and (*c*) a patient with normal ox brain prothrombin time (~46 s) designated Factor $IX_{Los\ Angeles}$ (IX_{LA}). This variant has been previously referred to as D.S. (19).

Reagents. Iodogen was obtained from Pierce Chemical Co., Rockford, IL. Rabbit brain thromboplastin, rabbit brain cephalin, and heparin were purchased from Sigma Chemical Co., St. Louis, MO. Antithrombin-III was obtained from Kabi Group, Inc., Greenwich, CT. $Na^{125}I$, $Na[^3H]$borohydride, and ^3H_2O were obtained from Amersham Corp., Arlington Heights, IL. Chemicals for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. Hereditary clotting factor-deficient plasmas were obtained from George King Biomedical, Overland Park, KS. Human Factor VIII concentrate (Koate) was kindly provided by Nazreen Pancham of Cutter Laboratories, Berkeley, CA. The preparation was activated with thrombin as described.[3] An ~12-fold increase in procoagulant activity was observed. Human brain tissue factor was a preparation obtained as described (20). All other chemicals were of the best commercially available grade.

Proteins. Human Factors IX and X were purified as described earlier (21). Human Factor VII was purified by the method of Bajaj et al. (22) except that the last step of purification, the preparative polyacrylamide gel electrophoresis was replaced by Sulfopropyl-Sephadex chromatography (23). Human Factor XI was purified by the procedure of Kurachi and Davie (24). Factor VIIa (22), Factor Xa (21) and Factor XIa (5) were prepared as described. Concentrations of purified proteins were determined spectrophotometrically using $E^{1\%}_{280}$ of 13.4 for Factor XI (24), 11.6 for Factor X (3), and 13.2 for Factor IX_N (6). A 13.2 value of $E^{1\%}_{280}$ was also used for each Factor IX variant protein since the amino acid compositions of Factor IX_N and each of the factor IX variant proteins were found to be very similar (Results).

Factor IX_{Normal} (Factor IX_N) and each of the variant Factor IX molecules were labeled with ^{125}I by using Bio-Rad Enzymobead reagent as described previously for prothrombin (25). Radiospecific activities of the preparations were: ^{125}I-Factor IX_N 3.7×10^9 cpm/mg, ^{125}I-Factor IX_{BmLE} 1.5×10^9 cpm/mg, ^{125}I-Factor IX_{LB} 3.5×10^9 cpm/mg, and ^{125}I-Factor IX_{LA} 3.1×10^9 cpm/mg. ^{125}I-Factor IX_N retained 95% of the biological activity of the unlabeled control. Factor VII was labeled with ^{125}I by using Pierce Iodogen reagent. The procedure followed was that outlined in the instruction manual supplied by the company. Specific activity of the preparation was 1.5×10^9 cpm/mg, and the labeled protein retained ~90% of the clotting activity of the nonlabeled control.

Tritium was incorporated into the sialic acid residues of Factor IX_N, variant Factor IX proteins, and Factor X by the general technique of Van Lenten and Ashwell (26). The procedure was slightly modified in that the reagent $NaIO_4$ was used at equimolar concentration to the sialic acid content of Factor X and at twofold molar concentration to the sialic acid content of Factor IX. A value of 12 for sialic acid residues per Factor X molecule and a value of 10 for sialic acid residues per Factor IX_N molecule was used (3). The same value of 10 for each Factor IX variant protein was assumed. Specific activities of the preparations were: ^3H-Factor IX_N 3.1×10^8 cpm/mg, ^3H-Factor IX_{BmLE} 2.1×10^8 cpm/mg, ^3H-Factor IX_{LB} 1.5×10^8 cpm/mg, ^3H-Factor IX_{LA} 1.9×10^8 cpm/mg, and ^3H-Factor X 1.3×10^9 cpm/mg. ^3H-Factor IX_N retained 84% and ^3H-Factor X retained 81% of the clotting activity of the nonlabeled controls.

Coagulation factor assays. Coagulation activities of Factors IX and X were measured as described (21). The clotting assay and the coupled amidolytic assay for Factor VII were performed as outlined by Seligsohn et al. (27) except that the human brain thromboplastin was replaced by rabbit brain thromboplastin (23). Ox brain prothrombin times were measured as described (18). A value for normal plasma was 41±3 s.

Electrophoresis. Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was performed according to the procedure of Weber and Osborn (28). The protein standards used to determine apparent molecular weight have been described (22). Nondenaturing disc gel electrophoresis was performed according to the procedure of Davis (29). ^3H and ^{125}I radioactivity profiles of $NaDodSO_4$ 1-mm gel slices were obtained as described (8).

Isoelectric focusing. Isoelectric focusing on acrylamide gels was carried out in 9.3 M urea as described earlier for prothrombin (25).

Amino acid composition. Amino acid analysis of Factor IX_N and each Factor IX variant protein was performed on a Spinco model 119 amino acid analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) equipped with a 20 mm cuvette and an Autolab Integrator. Protein samples were hydrolyzed with 6 M HCl in evacuated glass tubes for 24–72 h. Gla content of Factor IX_{BmLE} was estimated by hydrolysis of the samples with 2.5 M KOH in evacuated plastic tubes for 24 h (30), and the Gla content of Factors IX_{LA} and IX_{LB} was estimated by the amount of tritium incorporated specifically at the γ-carbon of Gla (31). Gla content of Factor IX_N was estimated by both methods (30, 31).

^3H-Activation peptide release assays. The rates of activation of ^3H-Factor IX_N and of ^3H-Factor IX variant molecules were measured by the activation peptide release assay of Zur and Nemerson (32) as described (5). Similarly, the rates of activation of ^3H-Factor X were measured from the activation peptide release as outlined by Silverberg et al. (33). The minor modifications for this assay were also the same as described for Factor IX (5). The concentrations of various reactants in the incubation mixtures are given in the legends to Fig. 3 (for Factor IX) and Fig. 7 (for Factor X).

Binding of antithrombin-III to ^{125}I-labeled cleaved normal Factor

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IX and variant Factor IX molecules. For these experiments, ^{125}I-Factor IX_N and each of the three ^{125}I-labeled Factor IX variant proteins were activated by incubating ^{125}I-labeled Factor IX_N or a variant protein (10 μg/ml) with purified Factor XIa (0.2 μg/ml) in 0.05 M imidazole-HCl, 0.15 M NaCl, 1 mg/ml bovine serum albumin, pH 7.5 (Im/NaCl/Alb) buffer containing 5 mM Ca^{2+} for 15 min at 37°C. The activation was stopped by adding 2 μl of 0.3 M Na_2EDTA and 1 μl of 1 M diisopropyl fluorophosphate to a 100-μl aliquot of each incubation mixture. (Human Factor IXa is not inhibited by diisopropyl fluorophosphate.) 10 μl of antithrombin-III/heparin solution was then added to each incubation tube to give a final concentration of 50 μg/ml for antithrombin-III and 10 U/ml for heparin. 10-μl aliquots were withdrawn from the incubation mixtures at different times and added to 50 μl of $NaDodSO_4$-gel protein buffer containing 5% 2-mercaptoethanol, and the samples were subsequently analyzed for ^{125}I-radioactivity profiles upon $NaDodSO_4$ gel electrophoresis (8). Since antithrombin-III binds to the heavy chain, H_β, of Factor IXa (6), we calculated the percentage of Factor IXa complexed to antithrombin-III (ATIII · H_β complex) as follows: (cpm in ATIII · H_β peak)/(cpm in ATIII · H_β peak + cpm in H_β peak) × 100.

Activation of Factor VII by the cleaved normal and variant Factor IX proteins. For studies dealing with the activation of Factor VII and of Factor X, cleaved normal and variant Factor IX proteins were obtained by the incubation of Factor IX preparations (100 μg/ml) with Factor XIa (0.2 μg/ml) for 30 min at 37°C. The buffer used was 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 containing 5 mM Ca^{2+}. Under these conditions Factor IX proteins were >90% cleaved as examined by $NaDodSO_4$ gel electrophoresis. The cleaved Factor IX proteins were placed on ice and used within 20 min. Since Factor XIa does not activate Factor VII or Factor X, it was not removed from Factor IXa preparations. The activations of Factor VII by the cleaved Factor IX proteins were carried out as described previously (10, 22). The incubation mixtures contained normal or variant Factor IXa preparations (2.8 μg/ml), ^{125}I-Factor VII (2.5 μg/ml), phospholipid (50 μM), and Ca^{2+} (5 mM). The buffer used was 0.05 M Tris-HCl, 0.15 M NaCl, 1 mg/ml bovine serum albumin (Tris/NaCl/Alb), pH 7.5 and both Factor VII and Factor IXa preparations were treated with an antisera to Factor X (10) to prevent possible activation of Factor VII by trace amounts of contaminating Factor Xa. The incubation temperature was 37°C and 10-μl aliquots were removed at different times and added to 90 μl of Tris/NaCl/Alb containing 6 mM Na_2EDTA. The samples were further diluted with Tris/NaCl/Alb and assayed for Factor VII activity both in the clotting (VIIc) and coupled amidolytic (VIIam) assays. The results were expressed as VIIc/VIIam ratio (10). Samples were also withdrawn from the incubation mixtures at different times for $NaDodSO_4$ gel electrophoresis and radioactivity profiles of the reduced samples were obtained as described (8). The fraction of Factor VII present in the activated form was calculated as follows: 1 − (cpm in unactivated Factor VII peak)/(total cpm in all peaks).

Neutralization of an anti-Factor IX_N monoclonal antibody by the variant Factor IX proteins. A monoclonal antibody that impairs the coagulant activity of normal Factor IXa through interference with its binding to activated Factor VIIIC, was prepared and purified as described.[3] The ability of Factor IX variant proteins to neutralize the inhibitory activity of the antibody was studied as follows: Factor IX_N (5 μg/ml in Tris/NaCl/Alb) or a mixture of Factor IX_N and a variant Factor IX protein (both at 5 μg/ml) was incubated with an equal volume of various concentrations (0–24 μg/ml) of monoclonal antibody for 30 min at room temperature. Samples were then placed on ice, diluted as needed and assayed for Factor IX clotting activity.

Isolation of variant Factor IX proteins. Plasma from hemophilia-B patients was obtained by plasmapheresis, with informed consent. The variant Factor IX proteins were isolated by the same method as described for normal Factor IX (21). Approximately 4–12 liters of plasma was used for each preparation and the steps of purification included adsorption of vitamin K-dependent proteins onto barium citrate, ammonium sulfate fractionation, DEAE-Sephadex chromatography, and heparin agarose affinity chromatography. An electroimmunoassay that used rabbit antisera to normal Factor IX (13) was used to monitor variant Factor IX proteins during isolation.

Results

Purification of variant Factor IX proteins. All of the three variant Factor IX proteins behaved very similar to normal Factor IX throughout the entire purification procedure. Fig. 1 shows the elution profile obtained on heparin agarose chromatography when Factor IX_{BmLE} plasma was used as the starting material. Peak A was eluted with 0.02 M citrate, 1 mM benzamidine, pH 7.5, and contained the major fraction, designated H-II_1 (21), of prothrombin. Peaks B and C were eluted after the application of sodium chloride gradient. Peak B contained the minor fraction, designated H-II_2 (21), of prothrombin and peak C contained Factor X. Peak D was eluted after the conclusion of the gradient and application of the 0.02 M citrate, 0.6 M NaCl, 1 mM benzamidine, pH 7.5, buffer. Peak D contained Factor IX_{BmLE}. Similar elution profiles were obtained when Factor IX_{LA} or Factor IX_{LB}

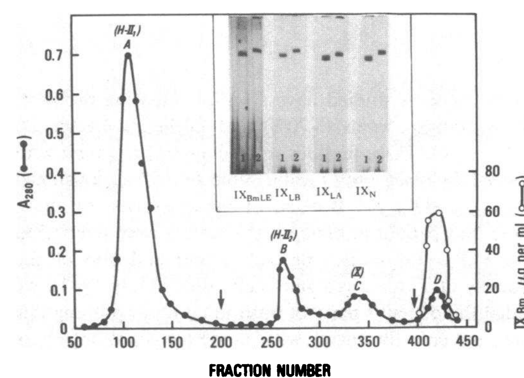

Figure 1. Purification of Factor IX_{BmLE} by heparin agarose column chromatography. Vitamin K dependent proteins from 3.94 liters of citrated Factor IX_{BmLE} plasma were adsorbed onto barium citrate, eluted and fractionated with ammonium sulfate, and chromatographed on the DEAE-Sephadex column as described (22). Protein in the DEAE-Sephadex fractions containing prothrombin, Factor X and Factor IX_{BmLE} was precipitated by the addition of solid ammonium sulfate to 80% saturation. The sample was dialyzed versus 0.02 M citrate, 1 mM benzamidine, pH 7.5, and applied to the heparin agarose column (2.5 × 45 cm) equilibrated with the same buffer (21). Approximately 200 mg of protein in 25 ml was applied to the column and 10-ml fractions before and 5-ml fractions after the application of the NaCl gradient were collected. A flow rate of 60 ml/h was maintained throughout. After the application of the sample, the column was washed with the initial buffer. Most of the prothrombin (H-II_1 peak A) was eluted with this buffer. A linear gradient (using 500 ml of buffer in each chamber) in NaCl (0.0–0.6 M) was then applied (left arrow). A minor fraction of prothrombin (H-II_2, peak B) and Factor X (peak C) were eluted in the gradient. At the completion of the gradient (right arrow), the column was developed with 0.02 M citrate, 0.6 M NaCl, 1 mM benzamidine, pH 7.5 buffer. Factor IX_{BmLE} (peak D) as monitored by an electroimmunoassay (13) was eluted with this buffer. The inset shows $NaDodSO_4$ gel electrophoretic analysis of the three variant Factor IX proteins and normal Factor IX purified by this method. In each case, gel 1 represents the unreduced sample and gel 2 represents the reduced sample. Approximately 10 μg of protein was applied to each gel.

plasma was used as the starting material. Approximately 0.75 mg (~30% yield) of a variant protein/liter of starting plasma was obtained.

The inset of Fig. 1 presents a photograph of $NaDodSO_4$ electrophoretic gels of the purified variant Factor IX proteins both before and after reduction of disulfide bonds. Each Factor IX variant was effectively homogenous and gave an apparent M_r of 61,000±2,000 by this method.

Physicochemical properties of variant Factor IX proteins. Each variant protein comigrated with normal Factor IX both in the $NaDodSO_4$ (28) and in the disc electrophoretic system of Davis (29). The amino acid compositions of each of the variant proteins were also very similar to that of normal Factor IX. These analyses were essentially the same as reported by other investigators for normal Factor IX (3) and are not presented again in this paper. Each variant protein also contained similar number of Gla residues. The average number of Gla calculated from three different analyses on alkaline hydrolysates (30) was 11.5 (10.2, 11.2, 13.1) for Factor IX_N and 11.4 (9.5, 11.8, 12.9) for Factor IX_{BmLE}. The average number of Gla calculated from tritium incorporation experiments (31) was 12.6 (10.3, 13.3, 14.1) for Factor IX_N, 11.2 (7.4, 11.3, 14.8) for Factor IX_{LA}, and 12.1 (11.4, 11.7, 13.3) for Factor IX_{LB}. Upon isoelectric focusing, normal and each variant Factor IX appeared as a broad protein band, and the isoelectric points (pI) ranged from 4.75 to 5.12 for Factor IX_N, 4.77 to 5.15 for Factor IX_{BmLE}, 4.88 to 5.25 for Factor IX_{LA}, and 4.86 to 5.3 for Factor IX_{LB}. From these data we conclude that each variant is very similar to Factor IX_N in its molecular weight, value of pI, amino acid composition, and number of Gla residues.

Cleavage of the variant Factor IX proteins by Factor XIa and by Factor VIIa. All of the three variant Factor IX proteins have been shown earlier to be normally cleaved by Factor XIa or by Factor VIIa (18, 19). To study accurate rates of cleavage of these molecules by a sensitive assay (32), we incorporated tritium into the sialic acid residues of these proteins (26). Identical conditions to those routinely used for normal Factor IX were used. The quantity of tritium incorporated into each variant protein was similar (specific radioactivities are given under methods) to that incorporated into normal Factor IX. Also, very similar ^3H-radioactivity profiles of partially activated samples of variant proteins and of normal Factor IX were obtained. A ^3H-radioactivity profile obtained by the partial activation of Factor IX_{BmLE} is depicted in Fig. 2 (solid circles). For comparison a ^3H-radioactivity profile obtained by the partial activation of normal Factor IX is also shown in Fig. 2 (open circles). From these profiles and from the profiles (not shown) of the partially activated other two ^3H-Factor IX variant proteins, it appears that very little tritium is incorporated into the heavy chain, H_β, of the variant Factor IXa molecules as previously observed for normal Factor IXa (8). Furthermore, complete activation of each variant protein as evaluated by $NaDodSO_4$ gel electrophoretic analysis resulted in ~35% (similar to that of normal Factor IX, [5]) of the total counts in the trichloroacetic acid (TCA) supernate. These data suggest that the carbohydrate distribution in each variant protein is very similar to that observed previously for normal Factor IX. Additionally, these data validate the use of the tritium release assay (5, 32) to monitor the rates of cleavages in variant Factor IX proteins. The rates of cleavage of purified variant proteins

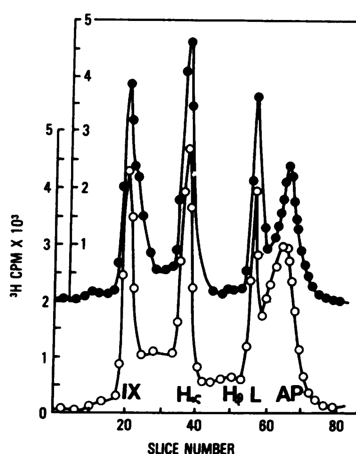

Figure 2. Reduced $NaDodSO_4$ radioactivity profiles of partially activated ^3H-Factor IX_N (*bottom*) and ^3H-Factor IX_{BmLE} (*top*). The incubation mixture contained ^3H-Factor IX_N or ^3H-Factor IX_{BmLE} 10 μg/ml, Factor XIa 32 ng/ml and Ca^{2+} 5 mM in Tris/NaCl/Alb buffer at 37°C. At different times 50-μl aliquots from the reaction mixtures were removed, inactivated by the addition of an equal volume of $NaDodSO_4$ protein buffer containing 10% 2-mercaptoethanol and subsequently subjected to $NaDodSO_4$ gel electrophoresis. The gels were sliced into 1-mm segments and the radioactivity determined as described previously (8). The profiles shown are of samples removed at 20 min.

as measured by this method were essentially the same as those observed for normal Factor IX when Factor XIa/Ca^{2+} (Fig. 3 *A*) or Factor VIIa/Ca^{2+}/tissue factor (Fig. 3 *B*) was used as the activating principle. These activation rates verify the conclusions of Østerud et al. (18, 19) drawn from the $NaDodSO_4$ gel electrophoretic data that the failure of these molecules to function in clotting does not stem from defective activation.

Functional activity of the cleaved variant Factor IX proteins. The experiments of this section examine the ability of the cleaved variant molecules to bind to antithrombin-III, to activate Factor VII, and to activate Factor X in the presence or absence of Factor VIII. The binding of antithrombin-III to the cleaved normal and variant Factor IX proteins was examined by incubating cleaved ^{125}I-labeled normal or variant Factor IX proteins with antithrombin-III/heparin solution. Samples were withdrawn at different times and analyzed for ^{125}I-radioactivity profiles obtained on reduced $NaDodSO_4$ gel electrophoresis (Methods). The profiles obtained with Factor IXa_N in the presence (top) or absence (bottom) of antithrombin-III incubated for 5 min are shown in Fig. 4 *A*. As expected, in

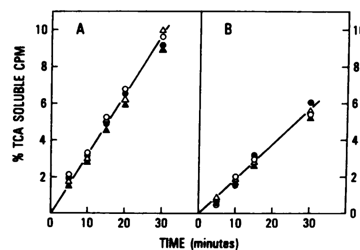

Figure 3. A comparison of the rate of cleavage of normal and variant Factor IX proteins by Factor XIa (*A*) and by Factor VIIa (*B*). The incubation mixtures contained 10 μg/ml of either ^3H-Factor IX_N or a ^3H-Factor IX variant protein in Tris/NaCl/Alb buffer containing 5 mM Ca^{2+} at 37°C. Additional components in the reaction mixtures were: for *A*, Factor XIa 32 ng/ml; for *B*, Factor VIIa 15 ng/ml plus tissue factor 12% by volume. Aliquots were withdrawn at different times and assayed for the amount of ^3H-activation peptide released. Approximately 35% of TCA soluble cpm represents 100% cleavage of Factor IX molecules. Symbols are: (○) ^3H-Factor IX_N, (●) ^3H-Factor IX_{BmLE}, (▲) ^3H-Factor IX_{LA}, (△) ^3H-Factor IX_{LB}.

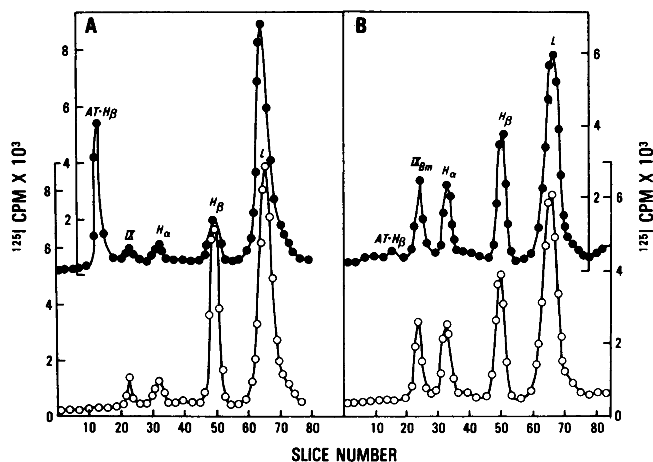

Figure 4. Reduced NaDodSO$_4$ radioactivity profiles of ^{125}I-Factor IXa$_N$ (*A*) and ^{125}I-Factor IXa$_{BmLE}$ (*B*) incubated with antithrombin-III/heparin solution. Samples were incubated with antithrombin-III (*top profile*) or with control buffer (*bottom profile*) for 5 min in the case of Factor IXa$_N$ and for 1 h in case of Factor IXa$_{BmLE}$. The exact conditions of incubation are given in Methods. AT·H$_\beta$ represents the complex of antithrombin-III and heavy chain of Factor IXa. Activation peptide does not contain tyrosine (2) and is not labeled with ^{125}I (8).

the presence of antithrombin-III, ^{125}I-radioactivity peak corresponding to the heavy chain, H$_\beta$, of Factor IXa$_N$ is substantially reduced and a new peak, AT·H$_\beta$, corresponding to a complex of antithrombin-III and H$_\beta$ has appeared. The radioactivity profiles of incubation mixtures of Factor IXa$_{BmLE}$ in the presence (top) or absence (bottom) of antithrombin-III incubated for 1 h are shown in Fig. 4 *B*. In contrast with the results obtained with Factor IXa$_N$ (Fig. 4 *A*), it is apparent from the profiles of Fig. 4 *B* that very little Factor IXa$_{BmLE}$ is bound to antithrombin-III even after 1 h of incubation.

In further experiments, the rates of formation of the ATIII·H$_\beta$ complex (Fig. 5) were calculated from the ^{125}I-radioactivity profiles obtained with activated Factor IX$_N$ and activated variant Factor IX proteins incubated for different times with antithrombin-III (Methods). When the rate of formation of ATIII·H$_\beta$ complex for Factor IXa$_N$ was set at 100% (Fig. 5, open circles), the relative rates for the variants were: ~0.5% for Factor IXa$_{BmLE}$ (closed circles) and ~6% for both Factor IXa$_{LA}$ (closed triangles) and Factor IXa$_{LB}$ (open triangles).

Functional activity of the cleaved variant Factor IX proteins was also examined by their ability to activate Factor VII.

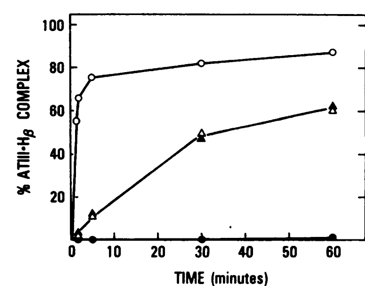

Figure 5. A comparison of the rates of a stable complex formation (ATIII·H$_\beta$) between antithrombin-III (ATIII) and the heavy chain (H$_\beta$) of cleaved normal and variant Factor IX proteins. Experimental details are given in Methods. ○, Factor IXa$_N$; ●, Factor IXa$_{BmLE}$; ▲, Factor IXa$_{LA}$; and △, Factor IXa$_{LB}$.

Results of a typical experiment are presented in Fig. 6. The incubation mixture contained ^{125}I-Factor VII, Ca^{2+}, PL, an antiserum to Factor X, and activated normal or a variant Factor IX. Activation of Factor VII was determined from the measurements of Factor VIIc/VIIam ratios (Fig. 6 *A*) and also from the ^{125}I-radioactivity profiles on NaDodSO$_4$ gels (Fig. 6 *B*). By both methods, the initial rates of activation of Factor VII were: 100% for Factor IXa$_N$, ~0.3% for Factor IXa$_{BmLE}$, and ~5–6% for both Factors IXa$_{LA}$ and IXa$_{LB}$.

The ability of the cleaved variant Factor IX proteins to activate Factor X was also investigated both in the absence and presence of thrombin activated Factor VIII. The reaction mixture in the absence of Factor VIII consisted of ^{3}H-Factor X, Ca^{2+}, PL, and normal Factor IXa or a variant Factor IXa preparation. Activation of Factor X in this system was very slow for both normal and variant Factor IXa preparations and only approximate rates could be obtained (Table I). Nonetheless, it was repeatedly observed (three experiments) that Factor X could not be activated by Factor IXa$_{BmLE}$ and the rates of activation of Factor X by Factors IXa$_{LA}$ and IXa$_{LB}$ were greatly reduced (~5–10%) compared with those obtained with Factor IXa$_N$ (Table I).

Reaction mixtures for activation of Factor X in the presence of Factor VIII consisted of ^{3}H-Factor X, Ca^{2+}, PL, thrombin activated Factor VIII/von Willebrand preparation, and normal Factor IXa or a variant Factor IXa protein. Fig. 7 presents plots of generation of Factor Xa under these conditions. It is apparent from these data that the rates of activation of Factor X by Factor IXa$_{LA}$ (closed triangles) and Factor IXa$_{LB}$ (open triangles) are ~6–7% of that obtained with Factor IXa$_N$ (open circles). Furthermore, activation of Factor X by Factor IXa$_{BmLE}$, although observable, could not be accurately measured.

These data demonstrate that Factor IXa$_{BmLE}$ has negligible activity (~0.2%), and both Factor IXa$_{LA}$ and Factor IXa$_{LB}$

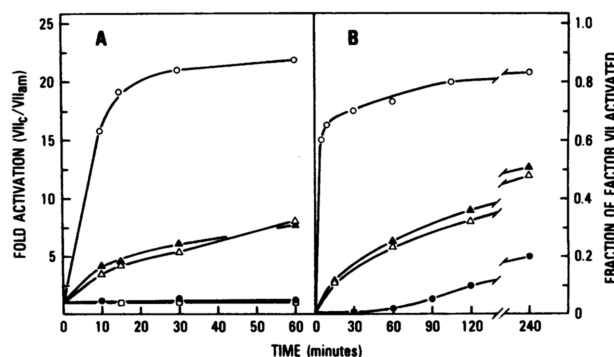

Figure 6. A comparison of the rates of activation of Factor VII by cleaved normal and variant Factor IX proteins. ^{125}I-Factor VII at 2.5 μg/ml was incubated with Factor IXa preparations at 2.8 μg/ml in the presence of 50 μM PL, 5 mM Ca^{2+} and an antiserum to Factor X (Methods). Samples were removed at different times and assayed for (*A*) Factor VII clotting versus coupled amidolytic activity (fold activation, VIIc/VIIam) and for (*B*) fraction of total ^{125}I-radioactivity present in Factor VIIa (fraction of Factor VII activated). For both *A* and *B* open circles represent Factor IXa$_N$, closed circles represent Factor IXa$_{BmLE}$, closed triangles represent Factor IXa$_{LA}$, and open triangles represent Factor IXa$_{LB}$. For *A* open squares represent a control reaction mixture in the absence of added Factor IXa. The VIIc/VIIam ratio for a fully activated Factor VII samples is ~25 (22).

*Table I. Activation of Factor X by Cleaved Normal and Variant Factor IX Preparations**

Activating principle	Rate of Factor Xa formation
	µg/ml/h
Factor IXa_N	~0.2
Factor IXa_{BmLE}	Not measurable
Factor IXa_{LA}	~0.01
Factor IXa_{LB}	~0.02

* ^{3}H-Factor X (10 µg/ml) in Tris/NaCl/Alb was incubated with normal or variant Factor IXa preparations (also 10 µg/ml), Ca^{2+} 5 mM, and PL 50 µM at 37°C. The amount of Factor Xa formed at different times (up to 3 h) was assayed by the release of ^{3}H-activation peptide (33).

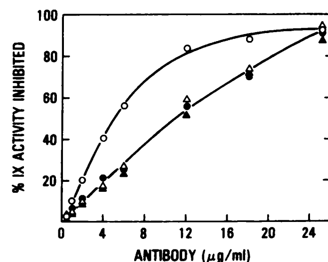

Figure 8. Evidence of binding of an anti-Factor IX_N monoclonal antibody to Factor IX variant proteins as measured by an antibody neutralization technique. 50-µl aliquots of Factor IX_N at 10 µg/ml were mixed with an equal volume of either buffer (open circles) or with a variant Factor IX protein also at 10 µg/ml (closed circles are for Factor IX_{BmLE}, closed triangles are for Factor IX_{LA} and open triangles are for Factor IX_{LB}). A 100-µl aliquot of the monoclonal antibody, ranging from 1 to 24 µg/ml, was then added to each tube. The samples were incubated for 30 min at room temperature and assayed for Factor IX activity in a clotting assay using Factor IX-deficient plasma.

have substantially reduced activity (~5–6%) in (*a*) binding to antithrombin-III, (*b*) activating Factor VII, and (*c*) activating Factor X in the presence or absence of Factor VIII.

Neutralization of an anti-Factor IX_N monoclonal antibody by the variant proteins. In these experiments, Factor IX_N was either diluted with buffer or with a variant Factor IX protein. The samples were then incubated with increasing concentrations of a monoclonal antibody (which interferes with the interaction of Factor IXa_N and activated Factor VIIIC)[3] for 30 min and assayed for Factor IX clotting activity. The data were plotted as percent Factor IX activity inhibited as a function of antibody concentration (Fig. 8). As can be seen from this figure, the concentration of the antibody needed to inhibit the same amount of Factor IX activity was twice as much when Factor IX_N was diluted with an equimolar concentration of a variant protein instead of the buffer. One may infer from this observation that normal and variant Factor IX proteins bind to this monoclonal antibody with the same affinity.

Discussion

We have characterized in detail three abnormal Factor IX variant molecules, namely Factor IX_{BmLE}, Factor IX_{LB}, and Factor IX_{LA}, with the objective to better understand structure-function relationships in normal Factor IX. Each of the three variants is a severe hemophilia-B patient and belongs to a different group according to the laboratory test results reported by Kasper et al. (13). Factor IX_{BmLE} plasma has a markedly prolonged ox brain prothrombin time and belongs to group I, Factor IX_{LB} plasma has a moderately prolonged ox brain prothrombin time and belongs to group II, and Factor IX_{LA} has normal ox brain prothrombin time and belongs to group III (13). Because prolongation of the ox brain prothrombin time is thought to be due to inhibition of the tissue factor dependent activation of Factor X (34), these variants were chosen with the hope that each variant may have a different molecular defect.

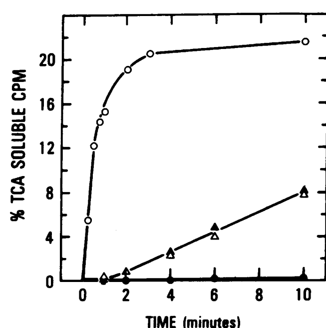

Figure 7. A comparison of the rates of activation of Factor X by cleaved normal and variant Factor IX proteins in the presence of Factor VIII. 20 coagulant U/ml of Factor VIII/von Willebrand protein were activated with 0.02 U/ml of thrombin for 3 min and the activated preparation was used immediately. Reaction mixtures contained 8 mM Ca^{2+}, 50 µM PL, 10 µg/ml ^{3}H-Factor X and 75% by volume of activated Factor VIII preparation. The final concentration of each Factor IXa preparation was 2 µg/ml. (We realize that only a fraction, probably the same for each variant, of Factor IXa will be bound to Factor VIII procoagulant activity under these conditions; however, concentration of 2 µg/ml for variant Factor IXa preparations was necessary to observe reproducible activation rates). Open circles represent Factor IXa_N, closed circles represent Factor IXa_{BmLE}, closed triangles represent Factor IXa_{LA} and open triangles represent Factor IXa_{LB}.

We establish that all three variant molecules are normal with respect to the molecular weights, amino acid compositions, number of Gla residues, range of isoelectric points and carbohydrate distribution. Although we did not carry out a complete carbohydrate analysis on these proteins, a similar amount of tritium incorporated into the sialic acid residues of these proteins and of Factor IX_N protein may suggest that each variant is also normal regarding its carbohydrate content. However, it should be noted that a point mutation resulting in a single amino acid substitution may not be detected by any of these analyses.

The variant molecules were also cleaved by Factor XIa/Ca^{2+}, and by Factor VIIa/Ca^{2+}/tissue factor at rates similar to those repeatedly observed for normal Factor IX (18, 19, present study). Since Ca^{2+} is needed for normal activation of Factor IX by Factor XIa, and Ca^{2+} and tissue factor (complex of PL and an apoprotein) are needed for normal activation of Factor IX by Factor VIIa, the proteolytic cleavage data along with the Gla data could be interpreted to mean that the Ca^{2+} and PL binding properties of the variant molecules are normal.

The cleaved normal Factor IX activates Factor X at an extremely slow rate in the absence of Factor VIII and at an accelerated rate in the presence of Factor VIII. Each cleaved variant molecule also showed an increased rate of activation of Factor X in the presence of Factor VIII (Fig. 7) compared with that obtained in its absence (Table I). Moreover, each variant was found to bind to an anti-Factor IX_N monoclonal antibody (that prevents the binding of Factor IXa to activated Factor VIIIC)[3] with an affinity similar to that observed for normal Factor IX (Fig. 8). The increased rate of activation of

Factor X in the presence of Factor VIII indicates that the Factor VIIIC binding site must be preserved in the variant proteins. The binding of the monoclonal antibody with a similar affinity suggests that the epitope that may be at or near the Factor VIIIC binding site is also preserved in these variant proteins.

The cleaved Factor IX_{BmLE} variant binds antithrombin-III and activates Factor VII or Factor X at insignificant rates (<0.5% of normal) and the cleaved Factors IX_{LA} and IX_{LB} bind antithrombin-III and activate Factor VII or Factor X at significantly reduced rates (~5–6% of normal). These data, presented in Figs. 5, 6, and 7, strongly indicate that the inability of the cleaved variant molecules to participate in clotting stems from defects in or around the active site serine residue.

To our knowledge, a total of five other Factor IX variant proteins has been investigated. Each variant described in the literature appears to be different from the variants described in the present report. Four of the variants (Factor $IX_{Zutphen}$, Factor $IX_{Chapel\ Hill}$, Factor $IX_{Alabama}$, and Factor $IX_{Eindhoven}$) reported in the literature have normal ox brain prothrombin times (14, 15, 35, 36). Factor $IX_{Zutphen}$ variant has an abnormally high molecular weight (~90,000) and an apparent reduced affinity for Ca^{2+} (14); additionally, the variant molecule could not be cleaved by Factor XIa (14). Factor $IX_{Chapel\ Hill}$ variant exhibits delayed activation with Factor XIa/Ca^{2+} (15, 16). This variant has been thoroughly investigated and is the only variant for which the precise amino acid substitution responsible for the molecular defect has been determined. The primary defect in this variant is the substitution of histidine for arginine at position 145; as a result of this, cleavage by Factor XIa at this peptide bond is precluded (17). Factor $IX_{Alabama}$ and Factor $IX_{Eindhoven}$ variants were cleaved normally by Factor XIa/Ca^{2+} and the cleaved molecule appears to be grossly similar to Factor IXa_N (35, 36). In preliminary studies, the low coagulant activity of these variants was attributed to the delayed activation of Factor X in the presence of Factor VIII (35, 36).

The fifth variant (Factor $IX_{Deventer}$) described in the literature has a markedly prolonged ox brain prothrombin time (37) as does our variant Factor IX_{BmLE}. The defect in Factor IX_{BmLE} is at or around the active site (present study). Whereas, the defect in the Factor $IX_{Deventer}$ appears to be at or around the Factor XIa cleavage site Arg^{180}-Val^{181} (37).

Hemophilia-B is a heterogenous disorder in which up to approximately one-third of the families possess abnormal Factor IX molecules (11–13). The plasmas from approximately one-fifth of the families with the abnormal molecules have strikingly prolonged ox brain prothrombin times (13, 38). These families have been placed into the hemophilia-Bm group (39). It would appear from studies of the two families (37, present study) that the patients in this group may not have the same molecular defect. Why these variant molecules (as compared to normal Factor IX) are stronger inhibitors (18) of the tissue factor dependent activation of Factor X is not known.

β-Hydroxy aspartic acid, a newly discovered amino acid in vitamin K-dependent proteins, is present both in bovine and human Factor IX (40). This amino acid has been implicated in binding of Ca^{2+} to bovine Factor IX (41). Due to the limited amount of variant proteins available and the difficulty in estimating β-hydroxy aspartic acid in proteins (40), we did not carry out an analysis for this amino acid. The content of this amino acid in other variant proteins is also not known.

Of interest is the fact that the catalytic efficiences of our two variants (Factor IX_{LA} and IX_{LB}) are apparently very similar. After cleavage, both of these variants possess ~5% of the activity of the cleaved normal Factor IX. These variants are from two unrelated kindreds and are classified into two different groups based upon their ox brain prothrombin times (13). In addition to the type of variant Factor IX molecule, the ox brain prothrombin time could be influenced by other clotting factors in plasma, especially Factor VII. In this regard, Factor IX_{LB} plasma had 0.94 U/ml of Factor VII clotting activity, and a slightly prolonged (~60 s) ox brain prothrombin time. By comparison, Factor IX_{LA} plasma had 1.15 U/ml (22% higher than Factor IX_{LB} plasma) of Factor VII clotting activity and normal ox brain prothrombin time (~46 s). It is thus not possible for us to state with certainty that the molecular defects in these molecules are different. The serine residue of the active site probably is intact since we have observed some proteolytic activity in these variants. The defect may be due to a substitution of the histidine and/or of the aspartic acid residue involved in the charge relay system similar to that described for trypsin (42). It is also possible that the defective catalysis is due to substitution of some other amino acid residue that helps bring the catalytic triad residues (histidine, serine, and aspartate) into close proximity. In contrast with these variants, a virtual absence of catalytic activity in Factor IX_{BmLE} may be due to the substitution of the active site serine itself.

Davie and co-workers (2, 6) have shown that the active site residues including histidine, serine, and aspartate are located on the heavy chain, H_β, of normal IXa. We plan to isolate the heavy chains of the cleaved normal and variant proteins using our monoclonal antibody.[3] Since there are two methionine residues in the heavy chain (2), we will prepare three CNBr fragments of each of the heavy chains. A comparison of the sequences of the three CNBr peptides obtained from normal and variant heavy chains should provide information about the putative residues essential for normal Factor IXa activity.

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